# Focus On: Genetically Altered Mouse Cancer Models

# Testing Compounds in Genetically Altered Mice

TOXICOLOGIC PATHOLOGY, vol. 26, no. 4, pp. 461–473, 1998


## The National Toxicology Program Evaluation of Genetically Altered Mice as Predictive Models for Identifying Carcinogens*

WILLIAM C. EASTIN, JOSEPH K. HASEMAN, JOEL F. MAHLER, AND JOHN R. BUCHER

*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709-2233*

ABSTRACT

National Institute of Environmental Health Sciences researchers are exploring the utility of genetically altered mice to study mechanisms of carcinogenesis. Two of these mouse models, the Tg.AC (carrier of an activated mouse H-*ras* oncogene) and the *p53*$^{+/-}$ (heterozygous for the wild-type tumor suppressor gene *Trp53*), have genetic alterations that appear to hasten their expression of chemically induced tumors. These 2 models have been proposed as a basis for new strategies for identifying chemical carcinogens and for assessing risk. The National Toxicology Program (NTP) is conducting a series of studies with these 2 genetically altered strains to further examine their strengths and weaknesses for identification of documented rodent and human carcinogens. In this first evaluation, candidates for study were drawn from the NTP historical database of 2-yr rodent carcinogenicity studies and the open literature (primarily for drugs). Results with this first set of 11 chemicals tested in genetically altered mice, compared with previous findings in the traditional 2-yr rodent assays and literature on human tumor findings, appear to support the premise advanced by Tennant et al that these models have the potential to serve as more rapid and less expensive test systems to identify carcinogens.

*Keywords.* Tg.AC mice; *p53*; carcinogenicity; toxicity; bioassay; National Toxicology Program; mutagenicity

### INTRODUCTION

Regulatory agencies evaluate all available toxicity and carcinogenicity data on specific chemicals or drugs to establish potential human risk through their use or their exposure. Two sources of the more critical data are human epidemiological studies and long-term rodent bioassays.

The most directly relevant data come from epidemiology studies, because they associate human health effects with environmental conditions. When a single environmental agent can be shown to be directly related to a specific health problem in an exposed population, the risk to humans for that agent is clear. Two fundamental limitations of epidemiology are that 1) it is difficult to relate a health problem to 1 environmental agent, since most exposures are poorly characterized and are mixtures of agents and that 2) there is an inherently low power to detect an effect. In addition, these studies are retrospective evaluations of hazards that may have already adversely impacted public health (1).

Advantages of animal studies include the capacity to evaluate single agents using an appropriate exposure route under controlled environmental conditions. In addition, these studies may be prospective. The 2-yr bioassay using both sexes of 2 rodent species is the most common protocol currently used to produce chemical carcinogenicity data. This protocol has become fairly standardized (21b, 24, 28, 29), and data from these studies are generally required by regulatory agencies to evaluate chemical toxicity from long-term exposure.

The National Toxicology Program (NTP) has provided a large component of the basic scientific data used by regulators to protect human health. Although still considered the best test model for obtaining carcinogenicity data, the 2-yr rodent bioassay uses large numbers of animals and normally takes up to 5 yr to complete and evaluate before the results can be reported.

In addition to generating safety assessment information, another NTP objective is to develop and validate improved test methods, including alternative test systems (22). The evaluation of genetically altered models to identify toxic agents is 1 approach that the NTP is taking to meet these objectives. The overall aim of these evaluations is to develop reliable methods for testing carcin-

* Address correspondence to: Dr. William Eastin, NIEHS, Mail Drop E1-02, P.O. Box 12233, Research Triangle Park, North Carolina 27709-2233; e-mail: Eastin@niehs.nih.gov.



0192-6233/98$3.00+$0.00

TABLE I.—Transgenic mice responses based on preliminary NIEHS results (25, 26).

| NTP study results | | | |
|---|---|---|---|
| *In vitro Salmonella* assay | 2-Yr rodent studies | Predicted transgenic mouse tumor responses | |
| | | Positive | Negative |
| + | + | $p53^{def}$ and Tg.AC | |
| − | + | Tg.AC | $p53^{def}$ |
| + | − | | $p53^{def}$ and Tg.AC |
| − | − | | $p53^{def}$ and Tg.AC |

ogenicity of chemical agents in a shorter period of time and using fewer animals. The impetus for the NTP evaluations came in part from the discussions at the "Mechanism-Based Toxicology in Cancer Risk Assessment: Implications for Research, Regulation, and Legislation" workshop held January 11–13, 1995 (12). At this workshop, Dr. Raymond Tennant presented preliminary data from studies under way (25, 26) in his and other National Institute for Environmental Health Sciences (NIEHS) laboratories that suggested that Tg.AC and heterozygous p53-deficient ($p53^{def}$) mice might be appropriate models to rapidly identify agents with carcinogenic potential. In general, workshop attendees thought that the preliminary results from genetically altered mice were promising and that further studies were required for complete evaluation of the models.

Preliminary results from NIEHS studies of Tg.AC and $p53^{def}$ mice (25, 26) combined with those from the *Salmonella* mutagenicity assay and NTP 2-yr rodent study databases indicated that (a) chemicals positive in *Salmonella* and showing cross-species carcinogenic potential in Fischer 344 (F344) rats and $B6C3F_1$ mice in the 2-yr studies would produce tumors in heterozygous $p53^{def}$ and Tg.AC mice; (b) chemicals negative in *Salmonella* but showing cross-species carcinogenic potential in F344 rats and $B6C3F_1$ mice in the 2-yr studies would be negative in the heterozygous $p53^{def}$ mice but positive in the Tg.AC mice; and (c) chemicals either positive or negative in *Salmonella* and showing no carcinogenic potential in F344 rats and $B6C3F_1$ mice in the 2-yr studies would be negative in heterozygous $p53^{def}$ and Tg.AC mice.

Assessment of chemicals in the *Salmonella* assay is a reasonably reliable way to discriminate mutagenic from nonmutagenic chemicals. In addition, the *Salmonella* assay has been shown to be the most predictive test of rodent carcinogenicity of 4 *in vitro* systems commonly used to identify a chemical's mutagenic potential (27). Although categorizing chemical carcinogens based solely on their mutagenic potential is an oversimplification of a complex process, the operational use of the categories in Table I was thought to provide a convenient framework for testing these models.

### *Models*

The Tg.AC line was produced in FVB/N mice by pronuclear injection of a v-Ha-*ras* transgene linked to a fetal ζ-globin promoter and an SV40 polyadenylation/splice sequence (11). Tg.AC mice behave like genetically initiated mice, rapidly developing epidermal papillomas in response to topical tumor promoter or carcinogen treatment. When the *in vitro Salmonella* assay is used as a measure of mutagenicity, Tg.AC mice appear to respond to genotoxic as well as nongenotoxic carcinogens (25, 26). While the response in carcinogen-treated mice is a dramatic increase in papillomas, untreated, singly housed Tg.AC mice do not usually develop any spontaneous tumors, and the histology of the skin is normal. It has been shown that the v-Ha-*ras* transgene is not significantly expressed in non–tumor-bearing Tg.AC tissues but is overexpressed in the proliferating component of benign and malignant tumors (3). Thus, it appears that expression of the transgene drives proliferation and subsequent tumor development in carcinogen-treated Tg.AC skin. There is also evidence for chemically induced transgene expression in the forestomach (R. Tennant, personal communication) and bone marrow of Tg.AC mice (3).

The $p53^{def}$ mouse line has 1 functional wild-type *p53* allele and 1 inactivated allele. The *p53* gene is critical to cell cycle control and DNA repair and is often found to be mutated or lost in human and rodent tumors (2). Mice with a single copy of the wild-type *p53* allele ($p53^{+/-}$ heterozygous) offer a single target for mutagens, a condition analogous to humans with some heritable forms of cancer. The functional hemizygous state should increase the probability for either loss of *p53* tumor suppressor function or gain of transforming activity by requiring only a single mutation (25, 26).

Heterozygous $p53^{def}$ mice are viable and show a low background tumor incidence up to almost 12 mo of age (4). This model is particularly attractive because in limited studies the same chemical-specific target organs appear to respond as in the 2-yr bioassay (25). These features, together with the observation that the *p53* gene has been shown to be altered in approximately 50% of human cancers, suggested that this model has the potential to identify mutagenic human carcinogens.

Studies completed to date suggest that both the Tg.AC and the $p53^{def}$ models are phenotypically stable, i.e., they do not develop tumors without a chemical or physical stimulus for up to the 12-mo time period during which they have been studied (25, 26). In previous studies done at NIEHS, all chemicals tested that produced papillomas in Tg.AC did so in less than 6 mo. A dose–response relationship has been observed with promoters and carcinogens studied to date, and in some studies mice have developed papillomas as early as 4 to 6 wk (25). $p53^{def}$ mice also respond within 6 mo. Thus, the exposure length for the current studies was set at 24 wk.

### *Chemicals*

Chemicals for the initial NTP studies were selected to determine whether (a) the models would detect human carcinogens; (b) the models would discriminate between carcinogens with different mechanisms of action; (c) the models would detect a carcinogen administered by more than 1 route of exposure; and (d) there is a sex difference in response, since only female mice were used in preliminary studies.

When NTP 2-yr rodent cancer data were not available

TABLE II.—Chemicals selected for studies.

| Chemical (proposed mechanism) | *Salmonella* and rodent bioassay | Selection rationale (bioassay target organs) |
|---|---|---|
| Human carcinogens | | |
| DES (hormonal) | SA− 2-yr+ | Known to induce cancers in humans and rodents at similar doses; acts through hormone receptor–mediated process (target organs: liver, ovary uterus/vagina, mammary) |
| Melphalan (genotoxic) | SA+ 2-yr+ | Potent carcinogen allowing eventual study of a wide dose range providing information concerning the relative sensitivity of the models compared to humans and the standard bioassay (target organs: spleen, thymus, bone marrow) |
| Cyclosporin A (immunosuppressive) | SA− 2-yr+ | May yield information on the utility of these models to identify cancer hazards that are not readily identified in rodent bioassays and that are thought to elicit cancer through mechanisms other than the direct alteration of DNA (target organs: spleen, immune system) |
| TCCD (receptor-mediated) | SA− 2-yr+ | Based on relative abilities of "dioxinlike" chemicals to induce gene products from activation of the Ah receptor; demonstration of a response in transgenics would provide a more convenient model to use in further studies of the potency for cancer induction in relation to other agents in this class (target organs of gavage study: thyroid, liver; target organs of topical study: integumentary) |
| Comparison of effects of route of administration | | |
| *N*-Methylolacrylamide | SA− 2-yr+ | Negative in $p53^{+/-}$ (25) chemical is well absorbed dermally and administration by this and an oral route will provide comparative information on systemic effects (target organs: harderian gland, liver, lung, and ovary; nerve damage in short-term studies) |
| Isomers | | |
| 2,4-DAT | SA+ 2-yr+ | To test the ability of the transgenic mouse models to discern differences between closely related chemicals with apparent differences in carcinogenic potential in the rodent bioassay; also, 2,4-DAT would provide an additional test of genotoxic chemicals that have caused a less-marked tumor response than the more potent carcinogens in the 2-yr bioassay (target organs of 2,4-DAT: liver, mammary gland; target organs of 2,6-DAT: none) |
| 2,6-DAT | SA+ 2-yr+ | |
| Noncarcinogens | | To examine hypothesis that rodent noncarcinogens will also be negative in the transgenic species regardless of mutagenic activity in *Salmonella* |
| *p*-Anisidine HCl | SA+ 2-yr− | Positive in SA; negative in $p53^{def}$ mice; mice were negative in 2-yr studies |
| Resorcinol | SA− 2-yr− | Negative in SA and 2-yr but positive in other genetox assays (target organs: none observed) |
| 8-Hydroxyquinoline | SA+ 2-yr− | Positive in SA, mouse lymphoma, but negative in CHO, *Drosophila,* and in 2-yr rodent study by the oral (dosed-feed) route (target organs: none) |
| Rotenone | SA− 2-yr− | Uniformly negative in SA, CHO cells, and *Drosophila*; has apparent anticarcinogenic effects (target organs in 2-yr study: none; target organs in 13-wk study: bone marrow, forestomach) |

Abbreviations: SA = *Salmonella* assay; SA+ = culture positive for *Salmonella*; SA− = culture negative for *Salmonella*; 2-yr+ = results positive for 2-yr rodent carcinogenicity studies; 2-yr− = results negative for 2-yr rodent carcinogenicity studies; CHO = Chinese hamster ovary cell mutation assay; see text for explanations of all other abbreviations.

for a particular chemical, the literature and medical reference books were used to establish dose levels and target organs. When possible, the NTP's historical 2-yr rodent carcinogenicity and *Salmonella* assay databases were used as resources for chemical selection. Candidate chemicals were placed into 1 of 4 groups based on results of the 2-yr and *Salmonella* studies. For the initial studies, only those chemicals with positive findings in both species and usually in both sexes were included in the category "positive carcinogenicity studies," and only those chemicals with negative results in both sexes of both species were considered "negative carcinogenicity studies." Chemicals were considered mutagenic if there were 1 or more positive findings in the battery of typically used *Salmonella* strains and nonmutagenic if there were only negative responses. Scientists from both the government and the private sector were solicited for comments on the NTP plan to evaluate the potential of the genetically altered mouse models and also on the set of chemicals selected for these initial studies.

The specific chemicals chosen for these studies are listed in Table II together with the results from other NTP sudies and the rationale for selection.

Because data existed for only a limited set of chemical exposure studies using these 2 transgenic models and because there was a limited availability of Tg.AC and $p53^{def}$ animals, it was decided that for the initial studies, more information would be gained about the utility of these models for identification of carcinogens by studying a number of chemicals at only 1 or 2 dose levels. The number of animals per test group was set at 15 to maximize the use of animals and to keep a reasonable number that would allow us to detect a difference in response between controls and exposed animals. The doses selected were

TABLE III.—Test agents, doses, and routes of exposure.

| Test agent (NTP studies) | Dose/Route/Frequency | |
|---|---|---|
| | Tg.AC | *p53*$^{+/-}$ |
| Human carcinogens | | |
| DES CAS No. 56-53-1 | Topical: 0 and 0.83 mg/kg in ethanol 2×/wk | sc injection 1,000 μg/kg in emulphor:ethanol:water (10:10:80) 2×/wk |
| Melphalan CAS No. 148-82-3 | Topical: 0, 8.3, and 25 mg/kg in methanol 1×/wk | ip injection 0, 0.3, and 1.5 mg/kg in propylene glycol 3×/wk |
| Cyclosporin A CAS No. 59865-13-3 | Gavage: 0, 10 and 25 mg/kg in olive oil:EtOH 5×/wk | Gavage: 0, 10, and 25 mg/kg in olive oil:EtOH 5×/wk |
| TCDD CAS No. 1746-01-6 | Topical: 0.166 μg/kg in acetone 3×/wk | Gavage: 0.25 μg in males and 1 μg/kg body weight for females, 2×/wk in corn oil:acetone (9:1) |
| Route comparison | | |
| *N*-Methylolacrylamide CAS No. 924-42-5 | Gavage: 0 and 50 mg/kg in water 5×/wk<br>Topical: 0 and 50 mg/kg in acetone 5×/wk | Studies conducted previously by NIEHS (25) |
| Negative controls | | |
| *p*-Anisidine HCl CAS No. 20265-97-8 | Topical: 0 and 133 mg/kg in ethanol 5×/wk | Studies conducted previously by NIEHS (25) |
| Resorcinol CAS No. 108-46-3 | Topical: 0 and 225 mg/kg in acetone 5×/wk | Gavage: 0 and 225 mg/kg in water 5×/wk |
| 8-Hydroxyquinoline CAS No. 148-24-3 | Topical: 0 and 225 mg/kg in acetone 5×/wk | Dosed-feed: 0 and 3,000 ppm |
| Rotenone CAS No. 83-79-4 | Topical: 166 mg/kg in acetone 5×/wk | Dosed-feed: 0 and 1,200 ppm |
| Isomer comparisons | | |
| 2,4-DAT CAS No. 95-80-7 | Topical: 0 and 30 mg/kg in ethanol (50:50) 5×/wk | Dosed-feed: 0 and 200 ppm |
| 2,6-DAT·2HCl CAS No. 15481-70-6 | Topical: 0 and 30 mg/kg in EtOH:$H_2O$ (50:50) 5×/wk | Dosed-feed: 0 and 200 ppm |

those used in prior 2-yr rodent studies or those reported in the literature to be carcinogenic to humans.

## MATERIALS AND METHODS

Both sexes of hemizygous Tg.AC and heterozygous *p53* mice were received from Taconic Farms (Germantown, NY) at 4–5 wk of age. Pedigreed homozygous Tg.AC males were mated to FVB/N females, and the hemizygous Tg.AC progeny were used for these studies. After a 10- to 14-day quarantine period, animals were assigned at random to treatment and control groups. Five animals per sex per strain were examined for disease and parasites prior to the start of study. Animals of each strain were randomly assigned to study groups on the basis of stratified body weights, using a computer-generated randomization program. Weight ranges did not exceed ±20% of the mean body weight for each sex. Mice were housed individually in polycarbonate cages and randomly distributed in stainless steel racks with automatic watering systems. All animals were uniquely identified by a tattoo placed at the base of the tail. Environmental conditions in the animal rooms were monitored continuously. At least 10 changes per hour of fresh filtered air were provided, and a 12-hr on/12-hr off light cycle of 40–50 foot candles placed 5 feet from the floor was maintained. Humidity and temperature were generally maintained at 50 ± 15% and within 22 ± 2°C during the quarantine and study periods. All animals received NIH-07 Open Formula Diet pellet form (Zeigler Brothers, Inc., Gardners, PA) *ad libitum* for all routes of exposure except dosed-feed studies in which mice received the meal form.

For each test agent, mouse model, dose level, and route of administration, 15 animals/sex received the subject chemical, and 13–30 control animals/sex received vehicle (topical, gavage, subcutaneous, and intraperitoneal studies) or untreated feed (dosed-feed studies) for 24 wk. Since the *N*-methylolacrylamide, resorcinol, 8-hydroxyquinoline, and rotenone topical studies in Tg.AC mice were started at the same time, they shared a common control group of 30 mice/sex. For the same reason, the 8-hydroxyquinoline, rotenone, 2,4-diaminotoluene (2,4-DAT), and 2,6-diaminotoluene (2,6-DAT) *p53*$^{def}$-mouse dietary studies shared a common control group of 30 mice/sex. In the diethylstilbestrol (DES) studies there were fewer than 15 control mice/sex; 1 control female and 2 control male *p53*$^{def}$ mice died on days 1–3 of the study and were not replaced. Ten additional animals were added to both sexes of dosed *p53*$^{def}$ mice, and 5 additional animals were added to the control groups in the single dose level resorcinol gavage study. This was done because resorcinol was one of the last studies to begin, because there were extra mice available, and because additional animals provided protection against early losses due to gavage errors.

For topical studies we used a constant concentration of the test agent, varying the volume to adjust the dosage for changes in body weight. Hair on the backs of the topically treated Tg.AC and cyclosporin A–gavaged Tg.AC mice was removed from the base of the tail to the interscapular region using animal clippers. For gavage and topical studies, the dosing schedule was up to 5 times per week (see Table III below for agent-specific frequency), weekdays only and exclusive of holidays, with all animals remaining on the treatment regimen until the day (within 24 hr) of sacrifice. For feed studies the animals received the subject chemical mixed with feed 7 days/wk for 24 consecutive wk.

All animals were observed in their cages twice daily, before 10:00 am and after 2:00 pm, including weekends and holidays, for moribundity, mortality, and clinical signs of toxicity. Body weights and clinical observations were recorded on study days 1 and 8 and weekly thereafter. Food consumption was recorded weekly in dosed-feed studies. Table III gives the dosing requirements and routes of exposure for individual test agents.

The original dosing schedule of 1 time per week for

TABLE IV.—Survival in mice receiving one of the known human carcinogens.

| Chemical agent | Male test groups | | | Female test groups | | |
|---|---|---|---|---|---|---|
| | Control | Low | High | Control | Low | High |
| DES | | | | | | |
| Tg.AC | 11/15 | — | 9/15 | 15/15 | — | 3/15* |
| *p53*$^{def}$ | 11/13 | — | 8/13 | 12/14 | — | 13/14 |
| Melphalan | | | | | | |
| Tg.AC | 12/14 | 6/15* | 3/15* | 12/15 | 15/15 | 4/15* |
| *p53*$^{def}$ | 13/15 | 2/15* | 0/15* | 13/15 | 2/15* | 1/15* |
| Cyclosporin A | | | | | | |
| Tg.AC | 14/15 | 13/15 | 10/15 | 13/15 | 10/15 | 13/15 |
| *p53*$^{def}$ | 14/15 | 15/15 | 15/15 | 15/15 | 15/15 | 15/15 |
| TCDD | | | | | | |
| Tg.AC | 13/15 | — | 13/15 | 11/15 | — | 13/15 |
| *p53*$^{def}$ | 14/15 | — | 15/15 | 13/15 | — | 15/15 |

* $p \leq 0.05$ vs controls (life table test).

melphalan had to be changed to once every 2 wk in the high-dose (25 mg/kg) male Tg.AC mice due to severe clinical signs and mortality observed after the first application. Treatment was stopped in both sexes of Tg.AC mice receiving the high dose of melphalan, at study week 4 for males and at week 5 for females. Intraperitoneal injections of melphalan to *p53*$^{def}$ mice were stopped after 17 wk on test due to decreased body weight gains, clinical signs of lethargy, and mortality.

### *Observations*

Mice were weighed individually on day 1 of test, after 7 days, and at weekly periods thereafter. The animals were observed twice daily, once in the early morning and once in the late afternoon, at least 6 hr apart (before 10:00 am and after 2:00 pm), including holidays and weekends, for signs of moribundity and death. Signs of toxicity noticed during these routine checks were recorded. Formal clinical observations including the status of skin at the site of application were made and recorded weekly.

### *Necropsy and Histopathologic Evaluation*

A complete necropsy was performed on all treated and control animals that either died or were sacrificed, and all tissues were saved in formalin. No organ weights were taken. Tissues were fixed in 10% neutral-buffered formalin, and those to be prepared for histology were embedded in paraffin, sectioned at 5–6 μm and stained with hematoxylin and eosin. Organs examined microscopically were the adrenal/pituitary/thyroid glands, kidney, liver, lung, lymph nodes (mandibular, mediastinal, mesenteric), ovary/uterus or testis/epididymis, skin (application site, topical), spleen, stomach, thymus, gross lesions, and chemical-specific suspect targets based on 2-yr study results (see Table II).

### *Statistical Evaluation*

For many chemicals, few if any animals died prior to the end of the study, and in these instances, differences in the incidence of neoplastic and nonneoplastic lesions were assessed by Fisher's exact test and Cochran-Armitage trend tests. For chemicals showing increased mortality, survival-adjusted statistical methods were used. These include logistic regression for incidental lesions and life table tests for rapidly fatal lesions (6).

## RESULTS

In the following sections, the survival and pathology data for treatment effects are presented for each study, with emphasis on the proliferative lesions observed. Morphological descriptions of the treatment-related lesions and neoplasms observed in control groups are detailed in Mahler et al (13). Unless otherwise stated, the incidence given for skin papillomas is that at the site of chemical application.

### *Human Carcinogens*

There was decreased survival for female Tg.AC mice receiving DES by topical application (Table IV). Terminal body weights were significantly higher for Tg.AC females (19%) and lower for both sexes of *p53*$^{def}$ mice (16–19%) receiving DES (by subcutaneous injection) compared with controls. The reason for this strain difference in body weight effects after exposure to DES is unclear. Topically applied DES produced an increased incidence of squamous cell papillomas in both sexes of Tg.AC mice. Treatment of *p53*$^{def}$ mice with DES did not cause any increases in tumor incidence (Table V).

TABLE V.—Responses in the Tg.AC and *p53*$^{def}$ mice after treatment with DES.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Tg.AC mice | | | | | | | |
| Neoplastic | | | | | | | |
| Skin | Squamous cell papilloma | 1/15 | — | 8/15* | 1/15 | — | 10/15* |
| Nonneoplastic | | | | | | | |
| Pituitary | Hyperplasia | 0/15 | — | 9/15* | 0/12 | — | 12/14* |
| Seminal vesicles | Atrophy | 0/0 | — | 5/5[a] | | | |
| Thymus | Atrophy | 2/14 | — | 7/12* | 0/15 | — | 10/13* |
| Uterus | Hyperplasia (cystic) | | | | 5/15 | — | 14/15* |
| *p53*$^{def}$ mice | | | | | | | |
| Neoplastic | | | | | | | |
| No response | | | | | | | |
| Nonneoplastic | | | | | | | |
| Ovary | Degeneration | | | | 0/14 | — | 14/14* |
| Uterus | Hydrometra | | | | 0/14 | — | 12/14* |

[a] These lesions were identified grossly.
* $p \leq 0.05$ vs controls.

TABLE VI.—Responses in the Tg.AC and *p53*$^{def}$ mice after treatment with melphalan.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Tg.AC mice | | | | | | | |
| Neoplastic | | | | | | | |
| Forestomach | Squamous cell papilloma | 0/14 | 8/15* | 2/15 | 4/15 | 14/15* | 7/15 |
| Lung | Alveolar/bronchiolar adenoma | 2/14 | 4/15 | 2/15 | 0/15 | 5/15* | 5/15* |
| | Alveolar/bronchiolar carcinoma | 0/14 | 0/15 | 0/15 | 0/15 | 1/15 | 0/15 |
| Skin | Squamous cell papilloma | 0/14 | 3/15* | 0/15 | 1/15 | 6/15* | 5/15* |
| Nonneoplastic | | | | | | | |
| Bone marrow | Cellular depletion | 0/14 | 5/15* | 7/15* | 0/15 | 0/15 | 4/15* |
| | Myeloid cell hyperplasia | 0/14 | 7/15* | 5/15* | 0/15 | 12/15* | 5/15* |
| Lung | Alveolar epithelium hyperplasia | 0/14 | 1/15 | 4/15* | 0/15 | 4/15* | 3/15* |
| Lymph node | Mandibular atrophy | 0/11 | 13/13* | 4/11* | 0/15 | 0/15 | 3/14 |
| | Mediastinal atrophy | 0/8 | 9/9* | 5/9* | 1/11 | 0/13 | 4/10 |
| | Mesenteric atrophy | 0/13 | 11/11* | 4/9* | 0/14 | 0/15 | 0/10 |
| Spleen | Lymphoid follicular cell depletion | 0/14 | 14/15* | 11/15* | 0/15 | 0/15 | 7/15* |
| | Hyperplasia | 0/14 | 13/15* | 5/15* | | | |
| Thymus | Atrophy | 1/13 | 8/11* | 6/8* | 2/14 | 1/15 | 4/13 |
| *p53*$^{def}$ mice | | | | | | | |
| Neoplastic | | | | | | | |
| Skin | Sarcoma | 0/15 | 10/15* | 1/15 | 1/15 | 3/15 | 0/15 |
| Malignant | Lymphoma | 1/15 | 1/15 | 9/15* | 1/15 | 0/15 | 3/15 |
| Nonneoplastic | | | | | | | |
| Thymus | Atrophy | 0/13 | 6/8* | 2/10 | 0/14 | 5/6* | 2/6* |

* $p \leq 0.05$ vs controls.

Survival was reduced in both sexes of mice receiving melphalan by topical application (Tg.AC) or by intraperitoneal injection (*p53*$^{def}$) except for low-dose (8.3 mg/kg) Tg.AC females (Table IV).

Three high-dose Tg.AC males were sacrificed in moribund condition in week 1 and in the weeks following, clinical signs indicated that this dose level was not well tolerated in males or females. Dosing of Tg.AC mice was stopped at week 4 for males and at week 5 for females. Only 3 males and 4 females survived until the end of the study. Tg.AC males treated with melphalan had terminal body weights 15–18% lower than male controls.

Male and female Tg.AC mice that received low-dose melphalan had an increased incidence of squamous cell papillomas in the skin and forestomach. Treated females had an increased incidence of alveolar/bronchiolar adenomas (Table VI). In high-dose males and females, the reduced survival may have limited the tumor response.

Terminal body weights of both sexes of *p53*$^{def}$ mice treated with melphalan were also 14–33% lower than controls. Intraperitoneal injection of melphalan to *p53*$^{def}$ mice increased the incidence of fibrosarcoma of the skin in low-dose (0.3 mg/kg) males and of malignant lymphomas in high-dose (1.5 mg/kg) males (Table VI). However, females appeared to be more sensitive than males to the toxicity of melphalan based on the earlier onset of mortalities both in high-dose (week 5 vs week 15) and in low-dose (week 11 vs week 17) groups, which may have limited a tumorigenic response in females (data not shown).

There were no differences in survival or terminal body weights of mice treated with cyclosporin A (Table IV). Cyclosporin A given by gavage to Tg.AC mice increased the incidence of malignant lymphomas in high-dose males and keratoacanthoma and squamous cell papillomas (combined) of the skin in high-dose females. The papillomas were observed on the dorsal skin at the site of hair removal, similar to the mice receiving other chemicals by topical application. There was also a significant ($p < 0.05$) trend toward increase in forestomach tumors in males receiving cyclosporin A (Table VII). There were no neoplastic or nonneoplastic effects of cyclosporin A in *p53*$^{def}$ mice.

Survival and terminal body weights of mice exposed topically (Tg.AC) or by gavage (*p53*$^{def}$ ) to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were not different than their respective controls (Table IV).

Female Tg.AC mice treated with TCDD had an increased incidence of keratoacanthoma, and both sexes had increased incidences of squamous cell papillomas of the skin at the site of application. There was also an increased incidence of nonneoplastic lesions in the livers of both sexes and on the skin of treated female Tg.AC mice (Table VIII).

There was no increase in the incidence of neoplasia in either sex of *p53*$^{def}$ mice treated with TCDD. The incidence of cytoplasmic vacuolization seen in the livers of both sexes was increased compared with that of controls (Table VIII).

### *Comparison of Routes of Exposure*

*N*-methylolacrylamide given either by topical application or gavage did not affect body weight gain or survival and did not produce tumors or significant nonneoplastic findings in Tg.AC mice of either sex (data not shown).

### *Isomers*

Survival was similar to controls for both sexes of mice receiving 2,4-DAT or 2,6-DAT in the Tg.AC (topical) and the *p53*$^{def}$ (dosed-feed) models (data not shown). Terminal body weights were similar to controls in both sexes of Tg.AC mice treated with 2,4-DAT or 2,6-DAT and in *p53*$^{def}$ mice receiving 2,6-DAT. However, terminal body weights of both sexes of *p53*$^{def}$ mice receiving 2,4-DAT

TABLE VII.—Responses in the Tg.AC and $p53^{def}$ mice after treatment with cyclosporin A.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Tg.AC mice (via gavage) | | | | | | | |
| Neoplastic | | | | | | | |
| Forestomach | Squamous cell papilloma | 0/15 | 0/15 | 4/15* | 1/15 | 2/15 | 4/15 |
| Skin | Keratoacanthoma | 0/15 | 0/15 | 0/15 | 0/15 | 1/15 | 2/15 |
| | Squamous cell papilloma | 1/15 | 1/15 | 0/15 | 0/15 | 1/15 | 2/15 |
| | Keratoacanthoma & squamous cell papilloma | 1/15 | 1/15 | 0/15 | 0/15 | 2/15 | 4/15* |
| All organs | Malignant lymphoma | 0/15 | 0/15 | 4/15* | 0/15 | 1/15 | 0/15 |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |
| $p53^{def}$ mice (via gavage) | | | | | | | |
| Neoplastic | | | | | | | |
| No response | | | | | | | |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |

* $p \leq 0.05$ vs controls.

were 25% (males) and 12% (females) lower than those of their controls (data not shown).

There were no statistically significant increases in neoplasms caused by exposure to either isomer using the Tg.AC (topical) or $p53^{def}$ (dosed-feed) models (Table IX). However, squamous cell carcinomas of the skin were observed in 2 male Tg.AC mice receiving 2,4-DAT. This lesion has not been observed in male Tg.AC historical controls. Also, a squamous cell papilloma of the skin was observed in 1 dosed male and in 2 dosed females. Taken collectively, this effect is considered to be chemically related. Malignant lymphoma was observed in 1 male and in 2 female $p53^{def}$ mice receiving 2,4-DAT (Table IX). These low incidences were considered to be suggestive but not conclusive evidence of a chemically related carcinogenic effect. Malignant lymphoma is a relatively uncommon tumor in $p53^{def}$ mice, occurring in approximately 2% of the control mice evaluated in this set of studies (2/108 in males; 2/109 in females). A larger sample size in the $p53^{def}$ 2,4-DAT study would be necessary to clarify whether or not the marginal increase in malignant lymphoma is truly chemically related.

*Noncarcinogens in 2-yr Bioassay*

Survival was not different from that of the controls in Tg.AC mice receiving topical applications of *p*-anisidine HCl, resorcinol, 8-hydroxyquinoline, or rotenone. Terminal body weights of males receiving rotenone were 14.5% lower than those of controls. The body weights of females receiving rotenone and males and females receiving the other 3 test agents were not different from those of the controls (data not shown).

*p*-Anisidine and 8-hydroxyquinoline were not associated with any increases in neoplastic or important nonneoplastic lesions in either model (data not shown).

Resorcinol produced a significantly increased incidence of squamous cell papillomas in both sexes at the site of application. There were also significant increases in nonneoplastic lesions in the skin of treated mice. Increases included hyperplasia in both sexes and hyperkeratosis and inflammation and sebaceous gland hyperplasia in males. Some of the nonneoplastic lesions could be secondary to the presence of papillomas (Table X).

Exposure of Tg.AC mice to rotenone caused a distinctive and rare response characterized by a proliferation of myeloid cells in hematopoietic tissues and by an infiltration into multiple tissues by mixtures of mature and immature leukocytes (Table X). Although milder lesions had features of inflammatory processes, more severe manifestations resembled leukemic infiltration. The collective term myelodysplasia was used to describe this disorder and is described in more detail by Mahler et al (13) in this issue. The most severely and consistently affected tissue was the liver; in all treated females and all but 1

TABLE VIII.—Responses in the Tg.AC and $p53^{def}$ mice after treatment with TCDD.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Tg.AC mice | | | | | | | |
| Neoplastic | | | | | | | |
| Skin | Keratoacanthoma | 0/14 | — | 1/15 | 0/15 | — | 4/15* |
| | Squamous cell papilloma | 0/14 | — | 8/15* | 1/15 | — | 15/15* |
| | Squamous cell carcinoma | 0/14 | — | 0/15 | 1/15 | — | 3/15 |
| Nonneoplastic | | | | | | | |
| Liver | Focal necrosis | 0/14 | — | 4/15* | 3/15 | — | 10/15* |
| | Hepatocyte vacuolization | 0/14 | — | 8/15* | 0/15 | — | 2/15 |
| $p53^{def}$ mice | | | | | | | |
| Neoplastic | | | | | | | |
| No response | | | | | | | |
| Nonneoplastic | | | | | | | |
| Liver | Hepatocyte vacuolization | 6/15 | — | 14/15* | 0/15 | — | 6/15* |

* $p \leq 0.05$ vs controls.

TABLE IX.—Responses in the Tg.AC and *p53*$^{def}$ mice after treatment with 2,4-DAT and 2,6-DAT.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Tg.AC mice | | | | | | | |
| 2,4-DAT | | | | | | | |
| Neoplastic | | | | | | | |
| Skin | Squamous cell carcinoma | 0/15 | — | 2/15 | 0/15 | — | 0/15 |
| | Squamous cell papilloma | 0/15 | — | 1/15 | 0/15 | — | 2/15 |
| | Squamous cell papilloma + carcinoma | 0/15 | — | 3/15 | 0/15 | — | 2/15 |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |
| 2,6-DAT | | | | | | | |
| Neoplastic | | | | | | | |
| No response | | | | | | | |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |
| *p53*$^{def}$ mice | | | | | | | |
| 2,4-DAT | | | | | | | |
| Neoplastic | | | | | | | |
| Multiple organs | Lymphoma, malignant | 0/30 | — | 1/15 | 0/30 | — | 2/15 |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |
| 2,6-DAT | | | | | | | |
| Neoplastic | | | | | | | |
| No response | | | | | | | |
| Nonneoplastic | | | | | | | |
| No response | | | | | | | |

treated male, the liver was a site of cellular infiltration. The white pulp of the spleen, kidney, and lung was also a frequent site of cellular infiltration characteristic of myelodysplasia. In the red pulp of the spleen and the bone marrow, there was often a prominent proliferation of granulocytic cells (granulocytic hyperplasia) in the hematopoietic tissue that was considered to be related to myelodysplasia. Likewise, lymphoid hyperplasia and plasma cell infiltration in the lymph nodes, often causing gross enlargement of the nodes, were also considered components of myelodysplasia. At the skin site of application there was mild squamous hyperplasia of the epidermis and a dermal inflammatory cell infiltration composed of a mixture of granulocytes, mononuclear cells, and mast cells. No skin tumors were observed at the site of application.

*p*-Anisidine has been previously tested in *p53*$^{def}$ mice in dosed-feed studies and was reported to be negative (25, 26).

Survival was not significantly different in either sex of *p53*$^{def}$ mice treated with resorcinol, 8-hydroxyquinoline, or rotenone compared with controls. Terminal body weights in both sexes of mice treated with resorcinol were similar to controls. However, there was a reduction in terminal body weights in both sexes of *p53*$^{def}$ mice treated with 8-hydroxyquinoline (males, 21.6%; females, 8.4%) and with rotenone (males, 33.8%; females, 16.4%). Resorcinol, 8-hydoxyquinoline, and rotenone did not in-

TABLE X.—Responses of Tg.AC mice to chemicals that were negative in the 2-yr bioassay.

| Tissue | Lesion | Male dose groups | | | Female dose groups | | |
|---|---|---|---|---|---|---|---|
| | | Control | Low | High | Control | Low | High |
| Resorcinol | | | | | | | |
| Neoplastic | | | | | | | |
| Skin | Squamous cell papillomas | 3/30 | — | 10/15* | 1/30 | — | 12/15* |
| Nonneoplastic | | | | | | | |
| Skin | Hyperkeratosis | 3/30 | — | 8/15* | 0/30 | — | 0/15 |
| | Hyperplasia | 3/30 | — | 14/15* | 0/30 | — | 8/15* |
| | Inflammation | 3/30 | — | 12/15* | 0/30 | — | 1/15 |
| | Sebaceous gland hyperplasia | 3/30 | — | 14/15* | 0/30 | — | 1/15 |
| Rotenone | | | | | | | |
| Neoplastic | | | | | | | |
| Multiple organs | Myelodysplasia | 0/30 | — | 14/15* | 0/30 | — | 15/15* |
| Nonneoplastic | | | | | | | |
| Liver | Bile duct degeneration | 0/30 | — | 7/15* | 0/30 | — | 3/15* |
| | Hyperplasia | 0/30 | — | 4/15* | 0/30 | — | 3/15* |
| Lymph (mandibular) | Hyperplasia | 0/29 | — | 8/14* | 3/29 | — | 8/15* |
| Pituitary | Hyperplasia | 0/28 | — | 4/15* | 0/28 | — | 0/14 |
| Skin | Sebaceous gland hyperplasia | 0/30 | — | 7/15* | 0/30 | — | 9/15* |
| | Hyperkeratosis | 0/30 | — | 14/15* | 0/30 | — | 15/15* |
| | Hyperplasia | 0/30 | — | 15/15* | 0/30 | — | 15/15* |
| | Inflammation | 0/30 | — | 15/15* | 0/30 | — | 15/15* |
| Testes | Germ. epithelial degeneration | 0/30 | — | 5/15* | | | |

* $p \leq 0.05$ vs controls.

crease the incidence of neoplastic or nonneoplastic lesions in either sex of *p53*$^{def}$ mice (data not shown).

## Discussion

The Tg.AC mouse model has been proposed for use in detecting genotoxic or nongenotoxic carcinogens through a skin papilloma response to chemicals applied to the skin. The ease of scoring this response through visual inspection and the wealth of information that can be gathered on the progression and multiplicity of these lesions make this model very attractive. One of the purposes of these experiments was to examine mice for internal tumors and to compare their response to those obtained with exposure to chemicals applied by other routes in order to determine the full range of potential applications of the model. Of the 7 chemicals found to give positive tumor responses in Tg.AC mice, 6 of these chemicals resulted in skin papillomas, and this included 1 response to a chemical (cyclosporin A) given by gavage. In addition, except for the 2,4-DAT study, mice that developed this lesion were observed to have multiple skin papillomas. However, 1 chemical, rotenone, an expected negative, produced only an unusual myelodysplasia. Two chemicals, cyclosporin A and melphalan, produced forestomach papillomas; lung tumors were seen with melphalan; and a malignant lymphoma was also observed with cyclosporin A. Thus, it would appear that at least a limited histopathologic evaluation of internal tissues is warranted for the Tg.AC mouse model.

With the *p53*$^{def}$ mouse, the experience to date suggests that all tissues may be at risk of developing tumors, and there is limited but good agreement between tumor sites in the 2-yr bioassays and those in the *p53*$^{def}$ mice (25). With the current set of chemicals, only melphalan-treated male mice showed a clearly positive response: an increase in malignant lymphomas and fibrosarcomas. It had been proposed that a simple tabulation of grossly visible tumors might be sufficient to establish a positive response in this model; however, the current results indicate that a histopathologic evaluation will be as important with this model as with the Tg.AC.

Little useful information could be garnered from these studies concerning the route of exposure comparisons because the oral and dermal studies with *N*-methylolacrylamide were both unexpectedly negative. However, the modest skin tumor response in the gavage studies with cyclosporin A suggests that skin tumors may still be an expected and useful end point even when the chemical is applied elsewhere, giving promise for the eventual use of these models when chemicals are applied through inhalation or by oral route. It is useful to examine the individual responses of these models to each chemical before considering the data sets as a whole.

### *Diethylstilbestrol*

DES is a synthetic nonsteroidal estrogen receptor agonist that has been used to treat dysmenorrhea and to prevent spontaneous abortion. DES was designated as a human carcinogen by the IARC (8) based on increased incidences of clear-cell adenocarcinoma of the vagina and cervix in women and the risk of testicular cancer in males exposed *in utero*. Studies of the possible effects of DES treatment during pregnancy on the subsequent development of breast cancer have all have shown an increased risk in exposed women. DES has been tested for carcinogenicity in mice, rats, hamsters, frogs, and squirrel monkeys, producing tumors principally in estrogen-responsive tissues. DES was not mutagenic in *Salmonella* (34).

Systemic exposure to DES was evident after topical exposure to Tg.AC mice. Treatment-related estrogenic effects occurred in reproductive organs (testes, seminal vesicle, and uterus), in the liver, and in the pituitary gland, consistent with estrogenic effects known to occur in mice. There was also an increased incidence of skin papillomas in both sexes of Tg.AC mice. The skin of both male and female mice has been shown to have estrogen receptors (23, 30). However, the relationship between receptor activation and skin papilloma development is not clear. It is worth noting that no nonneoplastic effects were observed in the skin at the site of DES application.

As expected for a carcinogen acting through a hormonal mechanism, DES did not increase the incidence of neoplasms in *p53*$^{def}$ mice, although nonneoplastic treatment-related observations included effects consistent with exogenous estrogen administration, i.e., an increase in the incidence of ovary degeneration and hydrometra in females. Seminal vesicle atrophy was noted in treated males.

### *Melphalan*

Melphalan is an alkylating agent used in the treatment of selective human proliferative diseases, including multiple myeloma. IARC (8) has classified melphalan as having sufficient evidence of carcinogenicity in humans and animals. The classification for humans was based on epidemiological studies of patients with ovarian carcinoma, multiple myeloma, or breast cancer who consistently showed very large excesses of acute nonlymphocytic leukemia in the decade following therapy with melphalan. In animals, melphalan has been tested in mice and rats by intraperitoneal injection, producing lymphosarcomas and a dose-related increase in the incidence of lung tumors in mice and an increase in peritoneal sarcomas in rats. Melphalan was positive in *Salmonella* (33) and in most other genetic toxicity assays in which it has been tested.

Melphalan applied topically to Tg.AC mice reduced survival and body weights in both sexes receiving the high dose and in male mice receiving the low dose. Treatment-related increases in incidence of squamous cell papillomas were observed in skin in females and in forestomach in both sexes, and alveolar/bronchiolar adenomas were observed in the lung in females. In humans, melphalan has been shown to cause bone marrow toxicity. This effect was also observed in treated Tg.AC mice in these studies.

Survival was reduced for melphalan-treated *p53*$^{def}$ mice. Terminal body weights were lower in low-dose males. Necropsy revealed that the propylene vehicle caused extensive adhesions in the abdominal cavity of treated and control mice. There was a significantly increased incidence of fibrosarcomas at the site of injection

in low-dose male mice and of malignant lymphoma in high-dose male mice. Nonneoplastic treatment-related responses included atrophy in the thymus of low-dose males and females and atrophy of the ovary in low- and high-dose females. Degeneration of the testes was observed in high-dose male mice. These nonneoplastic effects are similar to those observed in the treated Tg.AC mice and may represent effects secondary to severe toxicity.

*Cyclosporin A*

Cyclosporins are fungal products used to treat graft versus host reactions through their immunosuppressive action. IARC (9) has designated cyclosporin as carcinogenic to humans on the basis of case reports of lymphomas and Kaposi's sarcoma and cohort studies reporting a high incidence of lymphoma in organ transplant recipients. In animal studies, cyclosporin enhanced the development of leukemias in a strain of mice prone to this tumor and in mice initiated with whole body irradiation or *N*-methyl-*N*-nitrosourea; cyclosporin also enhanced the incidence of intestinal adenocarcinomas induced by *N*-methyl-*N*-nitrosourea in rats (9).

The results seen with the Tg.AC mouse are consistent with the findings of prior human and animal studies. Malignant lymphoma was significantly increased in high dose males, and erythrocytic leukemias were diagnosed in 1 male and 2 females in the mid-dose groups. It is not clear why no high-dose females developed lymphomas or leukemias, although this dosed group had an increase in keratoacanthoma/squamous cell papillomas of the skin. Forestomach tumors also occurred in 4 high-dose males. Although forestomach tumors are relatively common (7%) in the control database (13), the finding of a low increase in incidence of tumors of types or sites known to occur in Tg.AC mice is consistent with expectations of the influence of an immunosuppressive action in this model. No tumors were seen in the *p53*-deficient mice given cyclosporin. This is consistent with the negative genetic toxicity profile of the chemical (33) and suggests that immunosurveillance is not as important in suppressing the development of spontaneous tumors in this model as it is in mice with an activated *ras* gene.

The results with cyclosporin A in the Tg.AC mouse differed in dosed males and females. Skin tumors were clearly increased in females, and lymphomas were increased in males. This was the only instance in the current studies in which a fairly clear sex difference was seen in the site of tumor response. In most cases, sex differences in tumor response involved the strength of the response at a common tumor site.

*TCDD*

TCDD is a contaminant in the manufacture of certain chlorinated herbicides; it is produced in pulp and paper bleaching and during waste incineration. IARC (10) has determined TCDD to be a known human carcinogen. In rodent studies TCDD has induced tumors in the liver, the lung, the thyroid gland, the subcutis, the hard palate, the nasal turbinates, the hematopoietic system, and the tongue (10, 17a, 17b). Evidence from human studies suggests that there are increases in cancers for all sites combined rather than for any particular tissue site.

In the Tg.AC mouse, TCDD induced squamous cell papillomas and carcinomas as well as keratoacanthomas. The response was stronger in females. In some female animals there was also evidence of an inflammatory response at the site of dermal application. No tumors were induced in the $p53^{def}$ mice receiving TCDD by gavage. TCDD is considered to be a nongenotoxic carcinogen; thus, the pattern of tumor response in these genetically altered models is in agreement with expectations. No systemic tumors were induced by TCDD in the Tg.AC mouse. Although TCDD has been shown to be systemically available in mice receiving the chemical on the skin, no tissue measurements were performed in these studies. Skin tumors have been induced in nongenetically altered mice by oral and dermal administration of TCDD (9, 10, 33).

*N-Methylolacrylamide*

*N*-methylolacrylamide was selected as a chemical not mutagenic in *Salmonella* but with clear evidence of carcinogenicity in both sexes of mice in the liver, lung, harderian gland, and ovary. Rat studies were negative with much lower doses than those used for mice (20). Human carcinogenicity of *N*-methylolacrylamide has not been established. Previous gavage studies with the $p53^{def}$ mouse were negative (25). For the current study, the chemical was administered to the Tg.AC mouse by the oral or dermal route. The chemical is absorbed through the skin, and therefore systemic exposure was anticipated to occur following administration by either route.

The negative result with the Tg.AC mouse given *N*-methylolacrylamide by either the dermal or oral route represents a major failure of the model. In the 2-yr study in $B6C3F_1$ mice, the carcinogenic responses were robust at the 50 mg/kg dose and included tumors in the harderian gland and ovary, sites that generally respond to fairly strong multisite carcinogens. While it is possible that the Tg.AC mouse would respond to a higher dose, our experience with this model suggests that a similar dose sensitivity is typically expected between the Tg.AC and the conventional mouse bioassay. The absence of a response in the Tg.AC mouse model precludes any conclusions concerning the intended comparison of the patterns of tumors seen after administration via the different routes.

*DAT Isomers*

2,4-DAT and 2,6-DAT were included in this evaluation to test the ability of the genetically altered mouse models to discern differences between closely related mutagenic chemicals with apparent differences in carcinogenic potential in the rodent bioassay. Also, 2,4-DAT would provide an additional test of genotoxic chemicals that have caused a less-marked tumor response than the more potent carcinogens in the 2-yr bioassay.

2,4-DAT is a widely used industrial intermediate. Most of this chemical produced in the United States is converted to toluene diisocyanate for use in the synthesis of polyurethanes. In the NTP 2-yr dosed-feed studies, 2,4-DAT was carcinogenic in F344 rats, inducing hepatocel-

lular carcinomas or neoplastic nodules in both males and females and carcinomas or adenomas of the mammary gland in females. 2,4-DAT was also carcinogenic in female B6C3F$_1$ mice, inducing hepatocellular carcinomas. The incidence of lymphomas in the female mice suggested that these tumors may also have been related to administration of the test chemical (15). The IARC has classified 2,4-DAT as possibly carcinogenic to humans (8). This agent was mutagenic in *Salmonella* (7) but negative in the micronucleus assay (NTP, unpublished observations). In the current studies, 2,4-DAT reduced body weight gain in Tg.AC and *p53*$^{def}$ mice, a response similar to that seen in rats and mice in the 2-yr NTP bioassay. Two male Tg.AC topically treated mice had squamous cell carcinomas of the skin, a lesion not observed in any of the historical male controls. In addition, there were papillomas in 1 male and 2 females but none in vehicle controls. No nonneoplastic effects were observed for the skin in any treatment group of Tg.AC mice. The effect on the skin of treated mice was considered to be chemical related. Although no increased incidences of neoplasms were observed in *p53*$^{def}$ mice that could be clearly related to treatment with 2,4-DAT, it is noteworthy that malignant lymphoma, which was marginally increased in *p53*$^{def}$ mice receiving 2,4-DAT, also had an increased incidence in female B6C3F$_1$ mice receiving 2,4-DAT in the standard 2-yr bioassay (15). And based on the positive *Salmonella* (7) and 2-yr bioassay results, a positive response was predicted in *p53*$^{def}$ mice. Further study of 2,4-DAT is required in the *p53*$^{def}$ mouse to determine if this response occurred by chance or if it was related to 2,4-DAT exposure.

2,6-DAT is used as an intermediate in the production of dyes for furs and textiles and of flexible polyurethane foams and elastomers. 2,6-DAT dihydrochloride was not carcinogenic for male and female F344 rats or for male and female B6C3F$_1$ mice in the 2-yr dosed-feed studies (16). 2,6-DAT has not been evaluated by the IARC. This agent was positive in *Salmonella* (31) and was positive in the micronucleus assay (NTP, unpublished observations). 2,6-DAT dihydrochloride did not cause an increased tumor incidence in either the Tg.AC or *p53*$^{def}$ mice. This outcome is in agreement with the prediction for a chemical that was negative in the 2-yr bioassay.

*p-Anisidine*

*p*-Anisidine, although mutagenic in the *Salmonella* (33) assay, was predicted to be negative in both the Tg.AC and the *p53*$^{def}$ mouse assays because it was negative in rats and mice in the conventional 2-yr rodent studies (14). Negative results with *p*-anisidine in the *p53* assay were previously reported by Tennant et al (25), and the negative results with the Tg.AC model in this report are in agreement with these findings and with expectations.

*Resorcinol*

Resorcinol (1,3-benzenediol) is used in the manufacture of adhesives and dyes and as an ingredient in pharmaceutical preparations for the topical treatment of skin conditions. Resorcinol is known to produce skin sensitization in humans and mice. In addition, resorcinol has been used to produce skin peeling in humans (5).

Resorcinol was not mutagenic in *Salmonella* studies (7). There were no treatment-related increased incidences of neoplasms or nonneoplastic lesions in rats or mice administered resorcinol by gavage for 2 yr in the NTP studies (21a). IARC (8) concluded that resorcinol was not classifiable as to its carcinogenicity in humans.

In contrast to expectations, resorcinol given topically to Tg.AC mice induced a strong skin papilloma response. Nonneoplastic lesions (including hyperkeratosis, inflammation, and hyperplasia) were also observed at the site of application, but whether these were secondary to the presence of the papillomas or in some way contributed to the tumor response could not be discerned from these limited studies. No systemic treatment-related lesions were observed.

Resorcinol given by gavage to *p53*$^{def}$ mice caused no increased incidence of neoplasia, a response similar to that seen in the 2-yr gavage studies in rats and mice.

*8-Hydroxyquinoline*

8-Hydroxyquinoline is used as a bacteriostatic and fungistatic compound in disinfectants. It was positive in *Salmonella* assays (31), but there was no evidence of carcinogenicity for male and female rats or mice given 8-hydroxyquinoline in feed in the NTP 2-yr bioassay (18). The IARC (8) concluded that 8-hydroxyquinoline was "not classifiable as to its carcinogenicity in humans." There were no increased incidences of neoplasias in the topical studies with Tg.AC mice or the dosed-feed studies with *p53*$^{def}$ mice. Both results were consistent with expectations.

*Rotenone*

Rotenone is a natural insecticidal and piscicidal constituent of several plant species. There was no evidence of carcinogenic activity in female rats and male or female mice fed diets containing rotenone for 2 yr (19). Rotenone was not mutagenic in *Salmonella* (32).

Despite the extensive hyperplasia and inflammation seen in skin at the site of application, there were no papillomas observed in Tg.AC mice treated with rotenone. However, topical exposure of the Tg.AC mouse to rotenone produced a systemic disease diagnosed as myelodysplasia. Myelodysplasia is a term used to encompass a spectrum of morphological changes in these mice consisting of mixed features of inflammatory, hematopoietic, and neoplastic processes. Exposure to rotenone did not induce an increased incidence of neoplasms or a similar proliferative response in the hematopoietic system of *p53*$^{def}$ mice in these studies or in B6C3F$_1$ mice in the NTP 2-yr dosed-feed studies (19).

Myelodysplasia is a disorder whose etiology and biologic behavior are unclear, perhaps because of unique effect of rotenone on the v-Ha-*ras*/ζ-globin promoter transgene. Some chemicals have been shown to have an effect on the fetal hemoglobin gene (for example, hydroxyurea, which is used in the treatment of sickle cell anemia based on its ability to increase fetal hemoglobin synthesis). It is possible that rotenone is acting similarly on the ζ-glo-

TABLE XI.—Comparison of results with predictions.

| Chemical | Known effects | | Tg.AC | | $p53^{def}$ | |
|---|---|---|---|---|---|---|
| | SA | 2-Yr study | Predict | Actual | Predict | Actual |
| DES | − | + | + | + | − | − |
| Melphalan | + | + | + | + | + | + |
| Cyclosporin A | − | + | + | + | − | − |
| TCDD | − | + | + | + | − | − |
| *N*-Methylolacrylamide | − | + | + | − (topical), − (gavage) | − | −[a] |
| 2,4-DAT | + | + | + | + | + | − |
| 2,6-DAT | + | − | − | − | − | − |
| *p*-Anisidine | + | − | − | − | − | −[a] |
| Resorcinol | − | − | − | + | − | − |
| 8-OH-quinoline | + | − | − | − | − | − |
| Rotenone | − | − | − | + | − | − |

Abbreviation: predict = prediction.
[a] Data from Tennant et al (25).

bin promoter of the transgene in Tg.AC mice, resulting in enhanced expression of the mutated *ras*, which is morphologically expressed as the aberrant proliferation of hematopoietic cells seen in multiple tissues. Until more is known about the biology of this disorder, it should be considered a potentially neoplastic effect. Hansen et al (3) found transgene expression in untreated bone marrow and occasionally in spleen in which ζ-globin expression was detected, and they suggested that factors regulating ζ-globin expression are available in these organs. Whether that mechanism is involved in the effects seen with rotenone is unknown.

Table XI compares the results of the model evaluations made with predictions based on our collective knowledge of the mutagenic and carcinogenic properties of these chemicals. For the $p53^{def}$ model, 2 chemicals were predicted to be positive, melphalan and 2,4-DAT. Melphalan was clearly positive in the $p53^{def}$ mice. 2,4-DAT, although clearly mutagenic, is only a moderately potent carcinogen in conventional rodent cancer studies and did not give a clear carcinogenic response in the $p53^{def}$ mouse. Thus, studies in the $p53^{def}$ mouse produced the predicted outcome for 10 of 11 chemicals; for the 11th chemical (2,4-DAT), a larger study would be needed to clarify whether or not the marginal increase in malignant lymphoma was truly chemically related.

For the Tg.AC, 6 of the 11 chemicals were predicted to be positive, and 7 actually gave a carcinogenic response. The Tg.AC mouse model detected all 4 recognized human carcinogens (with putative mutagenic, hormonal, immunosuppressive, and receptor-mediated mechanisms). Negative responses were expected with 5 chemicals, and 3 of these gave no neoplastic response in the Tg.AC mouse. The 3 failures, i.e., the lack of a positive Tg.AC response with *N*-methylolacrylamide, the myelodysplasia response to rotenone, and the skin papilloma response to resorcinol, present clearly discordant responses in need of further study.

Overall, of the 22 possible predicted outcomes, 18 responses (82%) in the Tg.AC and $p53^{def}$ studies were in agreement. Two "misses" were false positives, 1 was equivocal, and in only 1 case was a clear chemical carcinogen not identified. It is possible that this failure may be due to the very restricted dose ranges used in these studies (equal to carcinogenic doses in conventional rodent studies or observations in humans) or that *N*-methylolacrylamide simply acts through a mechanism that does not involve either mutations of the *p53* gene or the expression of *ras*.

Further efforts are needed to address in more detail issues of dose response and ways in which the results of studies with genetically altered rodent models can fit into strategies for carcinogen identification and risk assessment. Nonetheless, the results of the present studies clearly support a continued effort to integrate these and perhaps other genetically altered mouse lines into routine safety assessment studies.

## REFERENCES


1. Doll R (1996). Nature and nurture: Possibilities for cancer control. *Carcinogenesis* 17: 177–184.
2. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CAJ, Butel JS, and Bradley A (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* 356: 215–221.
3. Hansen LA, Trempus CS, Mahler JF, and Tennant RW (1996). Association of tumor development with increased cellular proliferation and transgene overexpression, but not c-Ha-*ras* mutations, in v-Ha-*ras* transgenic TgAC mice. *Carcinogenesis* 17: 1825–1833.
4. Harvey M, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A, and Donehower LA (1993). Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat. Genet.* 5: 225–229.
5. Harvey SC (1980). Antimicrobial drugs. In: Remington's Pharmaceutical Sciences, 16th ed., A. Osol (ed). Mack Publishing Co., Easton, Pennsylvania, pp. 1099–1178.
6. Haseman JK (1995). Data analysis: Statistical analysis and use of historical control data. *Regul. Toxicol. Pharmacol.* 21: 52–59.
7. Haworth S, Lawlor T, Mortelmans K, Speck W, and Zeiger E (1983). Salmonella mutagenicity test results for 250 chemicals. *Environ. Mutagen.* 5(suppl. 1): 3–142.
8. International Agency for Research on Cancer (1987). Overall evaluations of carcinogenicity: An updating of IARC Monographs, Vol. 1 to 42, Suppl. 7. Lyon, France.
9. International Agency for Research on Cancer (1990). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans,* Vol. 50, *Pharmaceutical Drugs.* Lyon, France, pp. 77–114.
10. International Agency for Research on Cancer (1997). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans,* Vol. 69, *Polychlorinated Dibenzopara-dioxins and Polychlorinated Dibenzofurans.* Lyon, France, pp. 1–342.
11. Leder A, Kuo A, Cardiff RD, Sinn E, and Leder P (1990). v-Ha-*ras* transgene abrogates the initiation step in mouse skin tumori-

genesis: Effects of phorbol esters and retinoic acid. *Proc. Natl. Acad. Sci. USA* 87: 9178–9182.

12. Lucier GW (1996). Mechanism-based toxicology in cancer risk assessment: Implications for research, regulation and legislation. *Environ. Health Perspect.* 104: 84–88.
13. Mahler JF, Flagler ND, Malarkey DE, Mann PC, Haseman JK, and Eastin W (1998). Spontaneous and chemically induced proliferative lesions in Tg.AC transgenic and *p53*-heterozygous mice. *Toxicol. Pathol.* 26: 501–511 (this issue).
14. National Cancer Institute (1978). *Bioassay of p-Anisidine Hydrochloride for Possible Carcinogenicity* (CAS No 20265-97-8). Technical Report Series No. 116, U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland.
15. National Cancer Institute (1979). *Bioassay of 2,4-Diaminotoluene for Possible Carcinogenicity* (CAS No 95-80-7). Technical Report Series No. 162, U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland.
16. National Cancer Institute (1980). *Bioassay of 2,6-Toluenediamine Dihydrochloride for Possible Carcinogenicity* (CAS No 15481-70-6). Technical Report Series No. 200, U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland.

17a. National Toxicology Program (1982). *Carcinogenesis Bioassay of 2,3,7,8-Tetrachlorodibenzo-p-dioxin* (CAS No 1746-01-6) *in Swiss-Webster Mice (Dermal Study).* Technical Report Series No. 201, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.

17b. National Toxicology Program (1982). *Carcinogenesis Bioassay of 2,3,7,8-Tetrachlorodibenzo-p-dioxin* (CAS No 1746-01-6) *in Osborne-Mendel Rats and B6C3F1 Mice (Gavage Study).* Technical Report Series No. 209, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.

18. National Toxicology Program (1985). *Toxicology and Carcinogenesis Studies of 8-Hydroxyquinoline* (CAS No 148-24-3) *in F344/N Rats and B6C3F1 Mice (Feed Studies).* Technical Report Series No. 276, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.
19. National Toxicology Program (1988). *Toxicology and Carcinogenesis Studies of Rotenone* (CAS No 83-79-4) *in F344/N Rats and B6C3F1 Mice (Feed Studies).* Technical Report Series No. 320, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.
20. National Toxicology Program (1989). *Toxicology and Carcinogenesis Studies of N-Methylolacrylamide* (CAS No 924-42-5) *in F344/N Rats and B6C3F1 Mice.* Technical Report Series No. 352, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.

21a. National Toxicology Program (1992). *Toxicology and Carcinogenesis Studies of Resorcinol* (CAS No 108-46-3) *in F344 Rats and B6C3F1 Mice (Gavage Studies).* Technical Report Series No. 403, U.S. Department of Health and Human Services, Public Health Service, Bethesda, Maryland.

21b. National Toxicology Program (1992). *Specification for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program* (NTP). Research Triangle Park, North Carolina.

22. National Toxicology Program (1996). *National Toxicology Program Annual Plan for Fiscal Year 1996.* Research Triangle Park, North Carolina.
23. Oh H-S and Smart RC (1996). An estrogen receptor pathway regulates the telogen-anagen hair follicle transition and influences epidermal cell proliferation. *Proc. Natl. Acad. Sci.* 93: 12525–12530.
24. Organisation for Economic Co-operation and Development (1981). *OECD Guidelines for Testing of Chemicals.* Paris, France. ISBN 92-64-12221-4.
25. Tennant RW, French JE, and Spalding JW (1995). Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. *Environ. Health Perspect.* 103: 942–950.
26. Tennant RW, Spalding J, and French JE (1996). Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutat. Res.* 365: 119–127.
27. Tennant RW and Zieger E (1993). Genetic toxicology: Current status of methods of carcinogen identification. *Environ. Health Perspt.* 100: 307–315.
28. U.S. Environmental Protection Agency (1982). *Health Effects Test Guidelines.* EPA Technical Report No. 560/6-82-001. Washington, D.C.
29. U.S. Food and Drug Administration (1993). *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* (Draft: Redbook II). *Federal Register,* Vol. 58, No. 58, p. 16536–16537, Washington, D.C.
30. Uzuka M, Nakajima K, and Mori Y (1978). Estrogen receptor in the mouse skin. *Biochim. Biophys. Acta* 544: 329–337.
31. Zeiger E, Anderson B, Haworth S, Lawlor T, and Mortelmans K (1988). *Salmonella* mutagenicity tests, IV: Results from the testing of 300 chemicals. *Environ. Mol. Mutagen.* 11(suppl. 12): 1–158.
32. Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K, and Speck W (1987). *Salmonella* mutagenicity tests, III: Results from the testing of 225 chemicals. *Environ. Mol. Mutagen.* 9(suppl. 9): 1–109.
33. Zeiger E, Anderson B, Haworth S, and Mortelman K (1992). *Salmonella* mutagenecity tests, V: Results from the testing of 311 chemicals. *Environ. Mol. Mutagen.* 19(suppl. 21): 2–141.
34. Zeiger E and Haworth S (1985). Tests with a preincubation modification of the *Salmonella*/microsome assay. In: *Evaluation of Short-Term Tests for Carcinogens,* J Ashby, F de Serres, M Draper, M Ishidate Jr, B Margolin, B Matter, and M Shelby (eds). Elsevier, North Holland, Amsterdam, pp. 187–199.